

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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In re Application of:

Kjetil TASKÉN et al.

Conf. No.: 4681

Appln. No.: 09/428,458

Group Art Unit: 1635

Filed: April 29, 1998

Examiner: Schmidt, M.

For: USE OF IMMUNOMODULATING AGENTS

SECOND DECLARATION UNDER RULE 132

Assistant Commissioner
of Patents
P.O. Box. 1450
Arlington, Virginia 2231-1450

I, Kjetil TASKÉN, a Norwegian citizen of Brekketunet 11, N-1349, Rykkinn, Norway;

declare as follows:

1. I am an inventor on the present application. A first Declaration was filed in connection with this application on 19 June 2002. This second Declaration supplements the evidence provided in the first Declaration. I have reviewed the Office Action dated 6 May 2003 which issued on the above application, wherein the Examiner raised objections under 35 U.S.C. 112, first paragraph, that the specification does not enable performance of the invention as claimed and that Gjertsen et al teach the unpredictability of the use of cAMP antagonists in view of their differing abilities to antagonize the actions of cAMP on protein kinase A (PKA). Experiments have been carried out under my direction to illustrate the common antagonistic effect of a variety of Rp-cAMP analogues on PKA Type I α activity by two different assays which determine EC₅₀ values of various Rp-cAMP analogues.

2. The purpose of the following analyses was to illustrate the antagonistic effect of Rp-cAMPS analogues on Type I (RI α /C α) holoenzyme complexes of the cAMP-dependent protein kinase. The

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extent of the antagonistic effect was measured by determining the EC_{50} values using suppression or activation assays based on the so-called Cook assay.

3. The $RI\alpha$ holoenzyme complex for use in the experiments was prepared by overnight dialysis of PKA $RI\alpha$ and PKA $C\alpha$ in a molar ratio of 1.2 to 1.0. Three 1 l buffer changes (dialysis buffer: 20 mM MOPS pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 100 μ M ATP, 5 mM β -mercapto-ethanol) were carried out to remove the cAMP from the regulatory subunit.

4. The holoenzyme complex and the assay conditions were tested prior to conducting the analysis of the analogues as follows: Holoenzyme was diluted (dilution buffer: 100 mM MOPS pH 7.0; 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT) to a 1 μ M stock solution and tested for activity in the spectrophotometric Cook assay (assay mix: 100 mM MOPS pH 7.0, 10 mM $MgCl_2$, 1 mM phosphoenol pyruvate, 1 mM ATP, 200 μ M NADH, 1 mM DTT, 15 U/ml lactate dehydrogenase, 70 U/ml pyruvate kinase). The reaction was started by mixing 1 μ l 25 mM kemptide (PKA substrate, LRRASLG) (200 μ M final concentration of active peptide) to 1 μ l holoenzyme (10 nM final concentration) in 100 μ l total volume of assay mix. OD_{340} was monitored for 1 minute and relative activity of $C\alpha$ was plotted as the slope of OD-decay/minute. Only a small residual activity of $C\alpha$ (<8% of the activated complex) showed a nearly complete formation of inactive holoenzyme complex.

4. The activation constant of cAMP was determined by using increasing concentrations of cAMP in a 3 minute preincubation with 10 nM holoenzyme in the assay mix. The results are shown in Annex 1, Figure 1. EC_{50} denotes the concentration at which half-maximal activation was achieved in this method and was 88 nM.

5. For the assays, all Rp-cAMPS analogues were dissolved in dilution buffer with 20% DMSO to a final concentration of 10 mM and the concentrations were determined spectrophotometrically using molar extinction coefficients at λ_{max} . Further dilutions of the Rp-cAMPS analogues were prepared by repeated 1:10 fold dilutions in

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dilution buffer. Preferably 1 μ l but not more than 5 μ l of the test Rp-cAMPS analogue was added to the assay mix. Therefore the DMSO concentration in the final assay mix was not higher than 1%. The effect of DMSO on the assay enzymes was tested and the results showed that DMSO concentrations of 1% or more had no effect on the assay enzymes (ADP columns) or Holo RI α (C α , Holo RI α and CAMP activated Holo RI α columns).

6. *Activation assay:* Rp-cAMPS analogues were screened to determine whether they behaved as antagonists or agonists. All Rp-cAMPS analogues were screened in an activation assay using 10 nM Holo RI α and 10 μ M of each Rp-cAMPS analogue. None of the compounds behaved as agonists or partial agonists and did not activate PKA (data not shown).

7. *Suppression assay:* The antagonistic properties of the Rp-cAMPS analogues were characterized in detail using a suppression assay. 10 nM RI α holoenzyme was partially (80%) activated by addition of agonist - 1 μ M Sp-8-Br-cAMPS (in the assay mix for three minutes). The holoenzyme was then reconstituted by the addition of the test Rp-cAMPS analogue (increasing concentrations ranging from pM to mM concentrations) for an incubation time of five minutes, before starting the assay of enzyme activity using the substrate kemptide. EC₅₀ determinations were carried out for the Rp-cAMPS analogues by the Cook spectrophotometric assay (described above). At least 10 measurements in duplicates were performed per analogue. In this assay, antagonists act by blocking binding and activation of PKA by Sp-8-Br-cAMPS by competitive antagonism and result in a decrease in kinase activity.


8. Rp-8-CPT-cAMPS (i.e. chlorophenylthio), Rp-8-Br-cAMPS, Rp-8-Cl-cAMPS, Rp-8-piperidino-cAMPS and Rp-cAMPS itself without any modifications were tested. The EC₅₀ value which is the concentration at which the activation is reduced to half its maximum was assessed. As can be seen from Annex 2, Figures 2 to 6, all the tested compounds work as effective antagonists of the PKA type I α as assessed by this assay.

9. These results therefore show that a variety of cAMP analogues

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have antagonistic and not agonistic effects on the PKA Type I α enzyme. The presence and extent of antagonism is readily testable and was found to be present in all of the Rp-cAMP analogues described above. In view of the antagonistic properties of these analogues, a negative effect on the activity of PKA Type I α and hence the signalling pathways in which that enzyme is involved may be expected when using these analogues *in vitro* or *in vivo* in cells employing that signalling involving PKA Type I α . It is therefore fully expected that the *in vitro* and *in vivo* effects observed using other PKA Type I α antagonists (as shown for example in my first Declaration) will also be achieved using PKA Type I α antagonists such as those described in this Declaration in view of their comparable antagonistic properties.

10. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such wilful false statements may jeopardize the validity of the application and any patent issuing thereon.


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Kjetil Taskén

10/23/2003

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Date